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PARTIAL PURIFICATION OF HUMAN PROSTATIC 5α-REDUCTASE (3-OXO-5α-STEROID:NADP$^+$ 4-ENE-OXIDO-REDUCTASE; EC 1.3.1.22) IN A STABLE AND ACTIVE FORM

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Summary—Human hyperplastic prostate tissue was homogenised in high ionic strength buffer and the post nuclear homogenate was incubated with 0.8% octyl glucoside and bovine brain lipids. Dialysis of the resulting liposome suspension yielded a preparation in which 5α-reductase was active and stable for at least three weeks and showed an increase in specific activity ($V_{max} \pm SD = 48.9 \pm 7.4$ pmol DHT/mg protein/ml) over that of the starting homogenate ($V_{max} \pm SD = 5.6 \pm 1.5$ pmol DHT/mg protein/min) of 8.7 times.

INTRODUCTION

5α-Reductase is found in several tissues in the body and is responsible for the reduction of steroids at the 5α position using NADPH as a cofactor.

In the prostate, testosterone is converted to its active form, 5α-dihydrotestosterone (DHT), which is one of a number of factors regulating cell growth in the prostate.

The solubilisation and purification of 5α-reductase from the prostate has been a particularly intractable problem because of its extreme susceptibility to detergents and its very strong membrane association [1–3]. However, it is important that a way of overcoming this problem is found because the activity of this enzyme has been implicated in the aetiology of two widespread diseases: benign prostatic hyperplasia and prostatic carcinoma [4–8].

Although some progress has been made towards the solubilisation of 5α-reductase from human tissue employing various detergents [1–3], the recoveries of solubilised enzyme was relatively low. We describe here a method for transferring 5α-reductase from the nuclear membrane of human prostate tissue, where it seems to be localised [9], to lipid vesicles in the presence of detergent. This method is based on one described by Racker *et al.* [10].

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EXPERIMENTAL

Materials

Hyperplastic prostate tissue was obtained from patients undergoing retropubic prostatectomy and stored at −70°C until used. [1,2,6,7-^{3}H]-testosterone, (sp. act: 80 Ci/mM) and molecular weight standards for SDS–PAGE were obtained from Amersham International, Amersham, Bucks. Other chemicals were from Sigma, Poole, Dorset.

Tissue preparation

Prostate tissue was finely minced with scalpels in a Petri dish on ice and transferred to 10 vol of the following buffer at 0°C: 100 mM Tris, 100 mM sodium citrate, 100 mM potassium chloride, 1 mM EDTA, 15 mM β-mercaptoethanol, 20% glycerol, pH 7.4 (buffer B in reference [1]) containing aprotinin 10 μg/ml, leupeptin 10 μg/ml, Nα-*p*-tosyl-L-arginine methyl ester (TAME) 10 μg/ml, 5 mM NADPH and 1 μM testosterone, hereafter "buffer". This was homogenised on ice in an Ystral homogeniser (Scottish Scientific Instrument Centre, Edinburgh) setting 5, for 1 min, filtered through nylon mesh and centrifuged for 20 min at 1200 *g*, 4°C. The supernatant (post nuclear homogenate) was centrifuged for 1 h at 105,000 *g*, 4°C, in a Sorvall OTD65 ultracentrifuge, rotor Sorvall T865.1. The resultant pellets were rinsed briefly in buffer, pooled and resuspended in 20 ml buffer

with brief Ystral homogenisation yielding the prostate microsomal preparation; this was kept on ice while the nest solution was prepared. A chloroform:methanol solution (1:2) of bovine brain phospholipids (Sigma B3635) was evaporated to dryness under vacuum and then resuspended with sonication in an MSE Soniprep 150, probe displacement = 20 μm, frequency = 23 kHz at 0°C using 10 sonication cycles of 20 s on and 40 s off to avoid excessive heating, in a solution of n-octyl β-D-glucopyranoside in 20 ml buffer. This suspension was added directly to the prostate microsome preparation to give a final concentration of 5 mg/ml lipids and 0.8% w/v octyl glucoside which was incubated on ice for 1 h with occasional agitation. This was then transferred to Spectrapor tubing (molecular weight cut-off = 12,000–14,000) and dialysed against two changes of 41 50 mM Tris, 0.9% sodium chloride, 20% glycerol and 5 mM dithiothreitol, pH 7.2 at 4°C for 3 days. The resulting preparation was kept at 4°C and used as the source of 5α-reductase for enzyme kinetic studies, discontinuous SDS–PAGE and as an immunogen for BALB/c mice. To examine the pH-dependence of the enzyme, the above preparation was dialysed against 2-(*N*-morpholino) ethanesulphonic acid (MES), 0.9% NaCl, 20% glycerol, 5 mM dithiothreitol at pH 5.5 and 4°C.

Assays

5α-Reductase activity was measured by the reduction of [1,2,6,7-^{3}H]testosterone by the enzyme suspension in the presence of an NADPH generating system at 37°C for various times. Details of the assay have been described previously [1]. The reaction was stopped by putting the assay tubes on ice and adding excess unlabelled carrier steroids and ^{14}[C]-DHT. Metabolites were extracted with diethylether, separated by thin-layer chromatography and losses were measured from the recovery of ^{14}[C]-DHT. Blank enzyme assays consisted of a boiled preparation of 5α-reductase and were used for subtraction of background activity. Testosterone and phenylmethylsulphonyl fluoride (PMSF) were dissolved in ethanol at 100 times final concentration; ethanol alone was added in the same amount to controls and blanks.

Lipid suspensions were prepared by evaporating an appropriate amount of lipid to dryness from its solution in chloroform and suspending it in buffer by sonication.

Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Watford, Herts) with bovine serum albumin as the standard.

RESULTS

The protease inhibitors, leupeptin, TAME and aprotinin did preserve the activity of 5α-reductase compared to the control (Table 1). The addition of 2 mM PMSF abolished the activity of the enzyme, as did the presence of 0.5 or 1% octyl glucoside or 0.5 or 1% lauryl dimethylamine oxide (LDAO) regardless of the

Table 1. The effect of various detergents and protease inhibitors on the 5α-reductase activity of the microsomal preparations

	Detergent used				
	None	0.5% O.G.†	1% O.G.	0.5% LDAO‡	1% LDAO
Buffer	100*	—	—	—	—
Buffer + p.i.§	127	39	20	10	10
Buffer + p.i. + PMSF (2 mM)	8	9	10	9	10

*Results are expressed as % of the control 5α-reductase activity. All values are means of duplicate determinations.
†O.G. = Octyl glucoside.
‡LDAO = Lauryl dimethyl amine oxide.
§p.i. (protease inhibitors) = leupeptin, TAME and aprotinin, all 10 μg/ml.

Table 2. The effect of additives on 5α-reductase activity* in the presence and absence of 0.8% octyl glucoside or 1.5% LDAO†

Buffer additives	Control	Detergent used 0.8% Octyl glycoside	1.5% LDAO†
Buffer + p.i.‡	100*	7	0
Buffer + p.i. + phosphatidlyethanolamine (5 mg/ml)	102	61	1
Buffer + p.i. + cholesterol (1 mg/ml)	110	15	5
Buffer + p.i. + phosphatidlyethanolamine (5 mg/ml) + cholesterol (1 mg/ml)	114	72	2
Buffer + p.i. + sigma B3635 (5 mg/ml)	112	103	0

*Results are expressed as % of the 5α-reductase activity of microsomal suspensions in buffer. All values are means of duplicate determination.
†LDAO = Lauryl dimethyl amine oxide.
‡p.i. (protease inhibitors) = leupeptin, TAME and aprotinin, all 10 μg/ml.

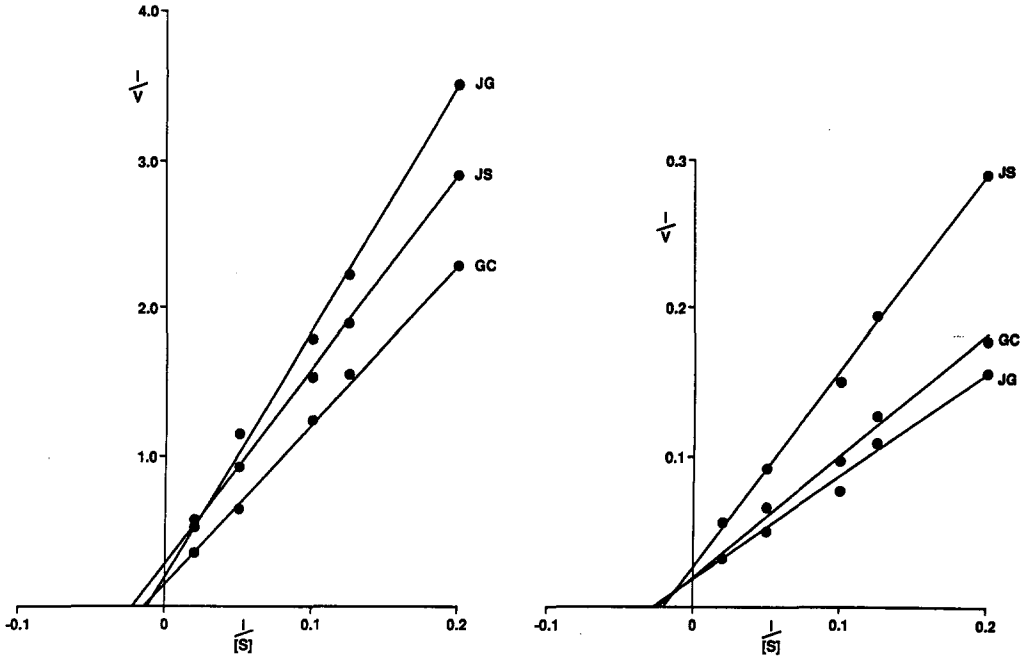

Fig. 1. Double reciprocal plot showing linear Michaelis–Menten kinetics of 5α-reductase in post-nuclear homogenate of human prostate gland (left) and liposome entrapped 5α-reductase (right), using concentrations (S) of testosterone of 5, 8, 10, 20 and 50 nM. V = rate of formation of dihydrotestosterone, pmol DHT/mg protein/min. For the sake of clarity the results from only three patients are shown.

presence of protease inhibitors. In view of these results it was decided to keep 5α-reductase in the presence of non-covalently interacting protease inhibitors in all future preparations.

Table 2 summarises the results of preparing 5α-reductase with various additives which may be expected to stabilise a membrane bound protein while attempting to solubilise it with detergent. Phosphatidylethanolamine had a moderate protective effect (activity about two-thirds of control value) while cholesterol was of little use in this respect either alone or in combination with phosphatidylethanolamine.

A suspension of whole brain lipids gave the best protection for 5α-reduction and even enhanced the activity slightly.

Of the various detergents tested for their ability to solubilise this enzyme, all denatured 5α-reductase in the absence of any lipid additive; activity was only maintained if octyl glucoside was used in conjunction with lipid to solubilise 5α-reductase in a functional conformation. LDAO is presented in Table 2 as an example for the other detergents used.

The kinetic parameters for five different prostate specimens were obtained from Michaelis–Menten plots of enzyme activity in the presence of [^{3}H]-testosterone at concentrations of 5, 8, 10, 20 and 50 nM. For the post nuclear homogenate, the apparent mean $K_m \pm$ SD was 67.1 ± 15.2 nM testosterone and apparent mean $V_{max} \pm$ SD was 5.6 ± 1.5 pmol DHT/mg protein/min ($n = 5$) whereas 5α-reductase solubilised in the presence of brain lipids showed an apparent $K_m \pm$ SD of 41.5 ± 6.5 nM testosterone and an apparent $V_{max} \pm$ SD of 48.9 ± 7.4 pmol DHT/mg protein/min ($n = 5$). These results demonstrate that the apparent K_m of the liposome entrapped enzyme has fallen slightly while the apparent V_{max} has increased by 8.7 times. The Michaelis–Menten plots for 3 randomly chosen patients are shown graphically in Fig. 1. The time-course of DHT formation by lipid-associated 5α-reductase was linear up to 140 min (Fig. 2).

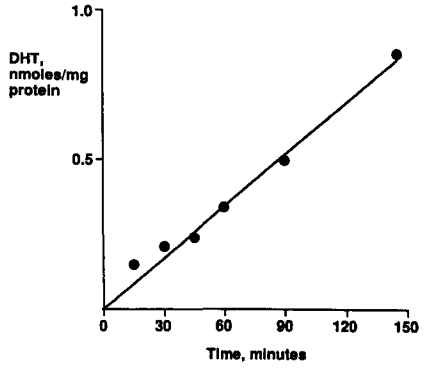

Fig. 2. Time-course of the formation of dihydrotestosterone (DHT) by liposome entrapped 5α-reductase obtained from one patient in the presence of 50 nM ^{3}H-testosterone.

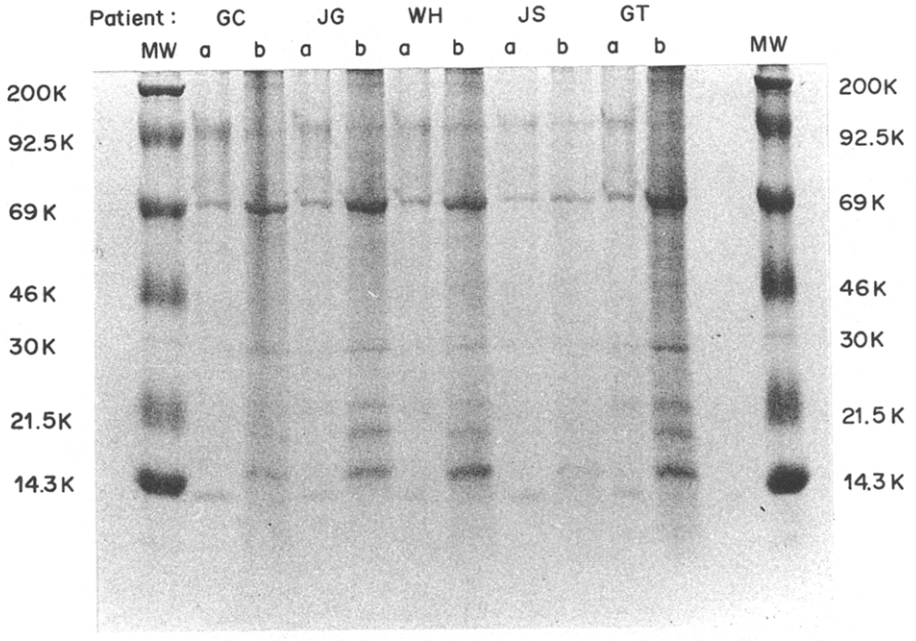

Fig. 3. Electrophoretic analysis on a 12.5% SDS–polyacrylamide gel under reducing conditions of proteins contained in either (a) the prostate microsomal preparation: or, (b) liposome entrapped 5α-reductase prepared from (a). Prostate tissue from five patients was used (top), molecular weight standards were run at both sides of the gel (14.3–200 K) and the gel was stained with Coomassie blue.

The proteins contained in the post nuclear homogenate and in the 5α-reductase preparation were separated by electrophoresis on a 12.5% polyacrylamide gel under reducing conditions and stained with Coomassie blue as shown in Fig. 3. The presence of brain lipids brought about a concentration of certain proteins since there were a number of relatively low molecular weight bands in the "b" lanes which were not obvious in the post nuclear homogenate, "a", lane. It was not possible to identify which of these bands was the 5α-reductase enzyme.

This enzyme preparation was inactive at pH 5.5 and loses approx. 50% of its activity in three weeks when stored at 4°C (data not shown).

DISCUSSION

For as long as a functional assay is needed to detect the presence of the enzyme steroid 5α-reductase, it will be necessary to keep this molecule in its active conformation. Since, in the prostate gland, it appears to be very strongly bound to the lipid environment of the nuclear membrane [3, 9] it was thought that any attempts to purify it by detergent extraction would need to be done in the presence of exogenous lipids, which might act as a replacement for the enzyme's natural niche. The choice of detergent is also crucial: a variety of different ones of diverse structures have been tested, for example, sodium cholate, digitonin and CHAPS, Lubrol PX, Triton X-100 and LDAO but only octyl glucoside preserved the activity of 5α-reductase and then only in the presence of lipid. This may be due to its structural similarly to a simple glycolipid, enabling it to exist in equilibrium between the lipid and bulk aqueous phases, thus facilitating transfer of 5α-reductase, and other proteins, into the multilamellar exogenous brain lipid. According to the distributors analysis, this lipid preparation (Sigma B3635) consists of, by weight, 10–15% phosphatidyl serine, 10–20% phosphatidyl ethanolamine, 10–15% sphingomyelin and 30–40% cerebroside, with the balance being made up of a mixture of saturated and unsaturated C_{16}–C_{22} free fatty acids. No information is available on the glycosylated lipid and ganglioside or cholesterol content. It is of interest that small amounts of phosphatidyl serine and phosphatidyl choline (the latter having particular acyl chain lengths) have been found to enhance the activity of microsomal testicular 5α-reductase [11].

The characteristics of the enzyme that we have "solubilized" in the presence of lipid are similar to those reported elsewhere, e.g. K_m and V_{max} values [7, 8, 12]. An acidic pH seems to inactivate the enzyme [13], perhaps by inducing a conformational change [14]. A modest purification

of about 9 times has been achieved, judging from the V_{max} of 5α-reductase in post-nuclear homogenate compared to that in association with brain lipids. Polyacrylamide gel electrophoresis of our enzyme preparation reveals a number of protein bands but it is not possible to assign one of these to the 5α-reductase molecule; however, the rat ventral prostate form of this enzyme has been predicted to have a molecular weight of 29 k [15].

In conclusion, now that a method for stabilising human prostatic steroid 5α-reductase in an active form has been found this should greatly facilitate its isolation so that a full understanding of its importance in normal physiology and in certain pathological conditions can be established.

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REFERENCES

1. Houston B., Chisholm G. D. and Habib F. K.: Solubilization of human prostatic 5α-reductase. *J. Steroid Biochem.* **22** (1985) 461–467.
2. Enderle-Schmitt U., Volck-Badouin E., Schmitt J. and Aumuller G.: Functional characteristics of nuclear 5α-reductase from rat ventral prostate. *J. Steroid Biochem.* **25** (1986) 209–217.
3. Enderle-Schmitt U., Neuhaus C. and Aumuller G.: Solubilization of nuclear steroid 5α-reductase from rat ventral prostate. *Biochim. Biophys. Acta* **987** (1989) 21–28.
4. Wilkin R. P., Bruchovsky N., Shnitka T. K., Rennie P. S. and Comeau T. L.: Stromal 5α-reductase activity is elevated in benign prostatic hyperplasia *Acta Endocr.* **94** (1980) 284–288.
5. Habib F. K., Busuttil A., Robinson R. A. and Chisholm G. D.: 5α-Reductase activity in human prostate cancer is related to the histological differentiation of the tumour. *Clin. Endocr.* **23** (1985) 431–438.
6. Hudson R. W. and Wherrett D.: Comparison of the nuclear 5α-reduction of testosterone and androstenedione in human prostatic carcinoma and benign prostatic hyperplasia. *J. Steroid Biochem.* **35** (1990) 231–236.
7. Bruchovsky N., Rennie P. S., Batzold F. H., Goldenberg S. L., Fletcher T. and McLoughlin G.: Kinetic parameters of 5α-reductase activity in stroma and epithelium of normal, hyperplastic, and carcinomatous human prostates. *J. Clin. Endocr. Metab.* **67** (1988) 806–816.
8. Tunn S., Hochstrate H., Grunwald I., Fluchter St.-H. and Krieg M.: Effect of aging on kinetic parameters of 5α-reductase in epithelium and stroma of normal and hyperplastic human prostate. *J. Clin. Endocr. Metab.* **67** (1988) 979–985.
9. Houston B., Chisholm G. D. and Habib F. K.: Evidence that human prostatic 5α-reductase is located exclusively in the nucleus. *FEBS* **185** (1985) 231–235.
10. Racker E., Violand B., O'Neal S., Alfonzo M. and Telford J.: Reconstitution, a way of biochemical research; some new approaches to membrane-bound enzymes. *Archs Biochem. Biophys.* **198** (1979) 470–477.
11. Ichihara K. and Tanaka C.: Partial characterization of testosterone 5α-reductase solubilized from rat testicular microsomes. *J. Steroid Biochem.* **33** (1989) 1249–1251.
12. Houston B., Chisholm G. D. and Habib F. K.: A kinetic analysis of the 5α-reductases from human prostate and liver. *Steroids* **49** (1987) 355–369.
13. Hudson R. W.: Comparison of nuclear 5α-reductase activities in the stromal and epithelial fractions of human prostatic tissue. *J. Steroid Biochem.* **26** (1987) 349–353.
14. Le Goff J. M., Martin P. M., Ojasoo T. and Raynaud J. P.: Non-Michaelian behaviour of 5α-reductase in human prostate. *J. Steroid Biochem.* **33** (1989) 155–163.
15. Andersson S., Bishop R. W. and Russell D. W.: Expression cloning and regulation of steroid 5α-reductase, an enzyme essential for male sexual differentiation. *J. Biol. Chem.* **264** (1989) 16249–16255.